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PRINCIPAL INVESTIGATOR: Lali K. Medina-Kauwe, Ph.D.

CONTRACTING ORGANIZATION: Cedars-Sinai Medical Center
Los Angeles, CA 90048

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Lali K. Medina-Kauwe, Ph.D.

Email: medinal@cshs.org

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14. ABSTRACT
The most significant findings here are that HerPBK10-siRNA complexes retain stability in whole serum and evade serum nuclease –mediated degradation of the siRNA, thus providing an encouraging prediction that the complex will be stable in vivo. We also show that HerPBK10-siRNA complexes induce targeted cell death to HER2+ but not HER2- cells in culture, suggesting that in vivo tumor targeting and cell death, as anticipated in the third year of this project, will be feasible. We show that the complex induces IFN-alpha secretion from HER2+ but not HER2- cells, which likely contributes to the mechanism of targeted cell death by these complexes. Interestingly, we also found that the HerPBK10 protein alone also induced a similar pattern of IFN-alpha secretion, and will be examining the contribution of the carrier protein and siRNA toward the targeted cell death observed here. Finally, we show that the siRNA carrier, HerPBK10, undergoes tumor-preferential accumulation in tumor-bearing mice, and preferentially avoids of normal tissues and organs.

15. SUBJECT TERMS
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INTRODUCTION:

The purpose of this research is to test the hypothesis that recombinant Ad5 capsid proteins targeted to HER2+ breast cancer induce tumor cell-specific death through: receptor-targeted binding and cell entry; siRNA-mediated "knock-down" of specific gene transcripts; and cytokine-mediated cytotoxicity. The main objective of this study is to demonstrate that heregulin-directed proteins target siRNA delivery to HER2+ cells in vitro and in a xenograft model of breast cancer in nude mice.

BODY:

Our first year of the project concluded with results showing that the HER2+ breast cancer –targeted carrier protein, HerPBK10, which we have previously used to target gene delivery (Medina-Kauwe et al., 2001) and corrole delivery (Agadjanian et al., 2006), stably assembles with synthetic anti-HER2 siRNA molecules and silences HER2 gene product formation in several HER2+ breast cancer cell lines.

Proposed plans for the second year of this project included transitioning from in vitro to in vivo studies. The success of experiments in vivo would depend on several factors, including stability of the conjugate in serum, specific targeting to HER2+ and not HER2- cells, and ability to target tumors in vivo. Here we report our results showing that the requirements to address these challenges have been met, and thus testing in vivo therapeutic efficacy, as planned in the final year of this project, will be feasible. Highlights of these results are summarized here.

Serum stability: HerPBK10 protects the siRNA from serum nucleases.

Nucleases present in serum and cells can degrade nucleic acid payloads and thus potentially reduce siRNA delivery (Medina-Kauwe et al., 2005). We examined the stability of siRNA conjugates in whole serum. HerPBK10-siRNA or siRNA alone were incubated in either 100% bovine serum or cell culture media (containing 10% serum) for 1h at 37°C and then assessed by agarose gel electrophoresis to examine the state of the siRNA. Ethidium bromide staining of the agarose gel identifies the siRNA molecular size and relative concentration. Ethidium bromide staining is nearly absent from samples in which the free siRNA was incubated with 100% serum, suggesting that the siRNA molecule is degraded by serum nucleases (**Fig. 1**). The same samples in which the siRNA is complexed with HerPBK10 shows that the siRNA molecule is intact and appears to be protected from serum nucleases (**Fig. 1**).

T7-transcribed siRNA induces higher breast cancer cell cytotoxicity than synthetic siRNA.

In the first year of this project, we reported our findings showing that HerPBK10-siRNA complexes induce cell-specific silencing of target HER2 gene transcripts. The siRNA used in those studies were obtained from a commercial source as pre-made, synthetic molecules (Dharmacon). As published findings indicate that T7 transcribed siRNA induce non-specific but potent cytokine-mediated cytotoxicity (Kim et al., 2004), we compared the cytotoxicity of T7 transcribed vs synthetic anti-HER2 siRNA on HER2+ cells. We acquired a 21 nucleotide (nt) synthetic anti-HER2 (ErbB2) siRNA and also produced a T7-transcribed molecule (Silencer

siRNA construction kit; Ambion) using the same sequence. Both were transfected into MDA-MB-435 human breast cancer cells in vitro at 1, 10, and 50 nM final siRNA concentrations and the cells assayed for survival at 96h after treatment. Whereas the synthetic siRNA had no effect on cell survival, the 10 and 50 nM concentrations induced substantial cell death (**Fig. 2A**). Similarly, when each siRNA is pre-assembled with HerPBK10, treated cells undergo significantly reduced cell survival after receiving the T7-transcribed siRNA compared to the synthetic siRNA (**Fig. 2B**).

SiRNA-facilitated cytotoxicity can be targeted to HER2+ but not HER2- cells by HerPBK10-mediated delivery.

As the cytotoxicity induced by T7-transcribed siRNA is thought to be non-sequence specific, we explored whether this effect could be targeted using our HerPBK10 protein. Our previous studies show that HerPBK10 binds and enters MDA-MB-435 but not MDA-MB-231 cells (Progress Report, Year 1), whereas commercial transfection reagents are not expected to be as discriminating with respect to receptor-targeting capacity. Several different doses of HerPBK10-siRNA complexes added to MDA-MB-435 (HER2+) and MDA-MB-231 (HER2-) cells produced a dose-dependent reduction in survival of the HER2+ cells (**Fig. 3A**) but had little to no effect on HER2- cells (**Fig. 3, A & B**). In contrast, siRNA delivered by a liposomal transfection reagent (Lipo-siHER2) significantly reduced the survival of both HER2+ and HER2- cells (**Fig. 3B**). Transfection reagent alone (Lipo), siRNA alone (siHER2), and HerPBK10 alone (0.03 mg/mL) had negligible effect on both cell lines (**Fig. 3B**).

Ligand-directed siRNA carrier vehicle, HerPBK10, targets HER2+ tumors in mice.

To get a sense of the targeting ability of the ligand *in vivo* and establish an index of *in vivo* targeting, we used a green fluorescent protein (GFP)-tagged ligand (GFP-Her), which has been used previously to demonstrate targeted receptor binding and endocytosis in HER2+ cells (Medina-Kauwe and Chen, 2002; Medina-Kauwe et al., 2000). Importantly, this ligand is identical to the 'Her' domain of HerPBK10. We established HER2+ tumors in 6-8 week female nude mice via bilateral flank injections of MDA-MB-435 cells. When the tumors reached 250-300 mm^3 (~3-4 weeks after tumor cell implant), 3 nmoles of GFP-Her was injected via the tail vein. Mock injected mice received saline alone. Indicated tissues were harvested at 3.5 h after injection and imaged for GFP using a Xenogen IVIS three-dimensional small-animal *in vivo* imaging system (Xenogen, Alameda, CA). Preferential accumulation of GFP fluorescence was detected in the tumors over the other tissues (**Fig. 4**). Low to negligible levels of fluorescence were detected in the liver and muscle, while GFP fluorescence was undetectable in the other tissues, including the heart (**Fig. 4**). Tissues from mock-treated animals showed no fluorescence (not shown). To further assess the in vivo targeting capacity of HerPBK10, tumor-bearing mice received systemic administration (as described earlier) of HerPBK10 labeled with a fluorescent molecule (S2Ga; (Agadjanian et al., 2006)). Mice were monitered for label circulation and biodistribution in real time using a small animal image acquisition system through the assistance of the Minimally Invasive Surgical Technologies Institute (MISTI) directed by Dr. Daniel Farkas at Cedars-Sinai Medical Center. Whereas the free, untargeted label exhibits distribution throughout most of the mouse (and, interestingly, appeared to be excluded from the tumors), labeled HerPBK10 exhibited preferential tumor accumulation in

comparison to other regions of the body (except for the injection site) (**Fig. 5**). The occasional high retention of injected material at the injection site of the tail vein is mostly due to technical complications in which some of the material is accidentally injected into the tail muscle rather than wholly in the vessel. This effect can be alleviated as injection technique improves.

The targeted siRNA complex as well as carrier protein alone induce interferon (IFN) –alpha secretion from HER2+ but not HER2- breast cancer cells.

The mechanism of T7-transcribed siRNA cytotoxicity is thought to be via the induced secretion of cytotoxic cytokines as a cellular response (Kim et al., 2004). To examine whether cytokines contribute to the targeted toxicity observed in Fig. 3, the media of HerPBK10-siRNA (T7-transcribed) –treated HER2+ MDA-MB-435 and HER2- MDA-MB-231 cells were collected and assayed by ELISA for interferons alpha and beta. Our findings show that IFN-alpha secretion from MDA-MB-435 cells is substantially elevated after treatment with HerPBK10-siRNA complex (at 10 and 50 nM siRNA dose) as well as HerPBK10 alone (at equivalent concentration to the 50 nM dose) (**Fig. 6A**). Whether the effect produced by the protein is elicited by receptor signaling remains to be seen, though it is interesting that despite the high IFN-alpha secretion in response to HerPBK10, the protein alone does not induce substantial cell death (**Fig. 3B**). MDA-MB-231 cells are little affected by either HerPBK10 or the complex (**Fig. 6A**). Both cell lines showed little to no sign of IFN-beta secretion regardless of treatment, though significant differences between HER2+ and HER2- cells were observed, especially after 10 nM HerPBK10-siRNA treatment (**Fig. 6B**). Given the overall low level of cytokine detected in the medium by all treatments, these differences are likely to have little relevance.

KEY RESEARCH ACCOMPLISHMENTS:

- Determination that HerBK10-siRNA complexes are stable in whole serum
- Determination that T7-transcribed siRNA induces enhanced toxicity to target cells over synthetic siRNA
- Demonstration that T7-transcribed siRNA delivered by HerPBK10 induces targeted toxicity to HER2+ but not HER2- cells
- Demonstration that the heregulin ligand of HerPBK10 preferentially accumulates in HER2+ tumors in vivo, and avoids organs and normal tissues
- Demonstration that HerPBK10 preferentially targets HER2+ tumors in mice using real time in vivo fluorescence imaging
- Demonstration that HerPBK10-siRNA complexes induce substantial secretion of IFN-alpha but not IFN-beta from MDA-MB-435 cells
- Determination that HerPBK10-siRNA complexes induce IFN-alpha secretion from HER2+ but not HER2- cells

REPORTABLE OUTCOMES:

- Presentation of research poster at the 2008 American Association of Cancer Research annual meeting (held in San Diego, CA)
- Presentation of research poster at the 2008 American Society of Gene Therapy annual meeting (held in Boston, MA)
- Upcoming presentation of research poster at the Department of Defense Era of Hope meeting (to be held this month in Baltimore, MD)

- Invited lecture entitled "Viral Capsid Proteins as Tumor-Targeted Nanotherapeutics", presented at the Department of Biomedical Sciences Molecular and Micro Imaging Symposium II, Cedars-Sinai Medical Center, March 12, 2008
- Invited lecture entitled "Tumor Targeting and Drug Trafficking", presented at the Neurosurgical Institute departmental seminar series, Cedars-Sinai Medical Center, April 21, 2008
- Preparation of manuscript for submission to Molecular Therapy

CONCLUSION:

The stability of HerPBK10-siRNA complexes in whole serum, specifically avoiding serum nuclease –mediated degradation of the siRNA, provides an encouraging prediction that the complex will be stable in vivo. The ability of HerPBK10-siRNA to induce targeted cell death to HER2+ but not HER2- cells suggests that in vivo tumor targeting and cell death, as anticipated in the third year of this project, will be feasible. As the targeted complex delivers T7-transcribed siRNA, it is possible that the mechanism of cell death is the targeted induction of cytokines. Indeed, we have shown that the complex induces IFN-alpha from HER2+ but not HER2- cells. However, the HerPBK10 protein alone also induced a similar pattern of IFN-alpha secretion. While it is possible that cytokine secretion may merely be a corresponding event associated with heregulin receptor binding, or may even result from receptor signaling, these findings do not correspond to the specific cytotoxicity observed from HerPBK10-siRNA but not HerPBK10 treatment. Therefore, the targeted cell death may be due to target transcript silencing by the siRNA molecule, or perhaps may result from the combination of effects elicited by both cell binding and entry of HerPBK10, and delivery of the siRNA. Follow-up experiments using a scrambled sequence will help determine whether silencing contributes to the targeted cell death observed here. It is also possible that the intersection of both ligand-induced signaling and cytokine induction synergistically facilitate the targeted cell death induced by HerPBK10-siRNA but not HerPBK10 alone or siRNA alone. Use of cytokine-blocking antibodies may further help specify the mechanism of targeted cell death observed here. Finally, the tumor-preferential accumulation of HerPBK10 in tumor-bearing mice, and preferential avoidance of normal tissues and organs, suggests that the carrier protein possesses the capacity to target siRNA to HER2+ tumor cells in vivo.

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FIGURES

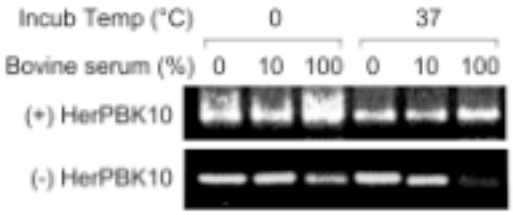

Fig 1. HerPBK10 protects siRNA in serum. Free siRNA alone (60 pmoles) or pre-incubated with HerPBK10 (2 ug, for 30 min at RT) was incubated in either complete media (10% bovine serum) or whole (100%) serum at 37°C for 1h, then assessed by agarose gel electrophoresis.

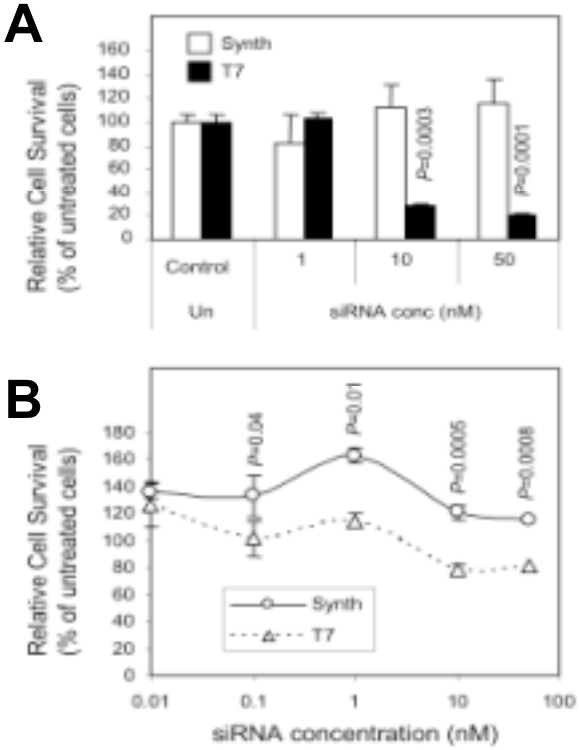

Fig 2. Effect of synthetic (Synth) vs T7-polymerized (T7) siRNA on cell survival. Indicated concentrations of anti-HER2 siRNA were transfected into MDA-MB-435 cells using (**A**) a commercial transfection agent (RNAimax) or (**B**) HerPBK10 (pre-assembled with siRNA at 4:1 charge ratio siRNA:protein), and assayed for cell survival using crystal violet stain at 96h after treatment. HerPBK10-siRNA complexes were assembled by co-incubating siRNA and HerPBK10 in 100mM Hepes in Optimem I buffer at RT for 30 min, then free siRNA removed by ultrafiltration through a 100 mwco membrane before adding to cells. Control cells were incubated in 100mM HEPES/Optimem I buffer alone. Un, untreated cells. P values reflect differences between synthetic vs T7 siRNA. Significant differences determined by 2-tailed t tests.

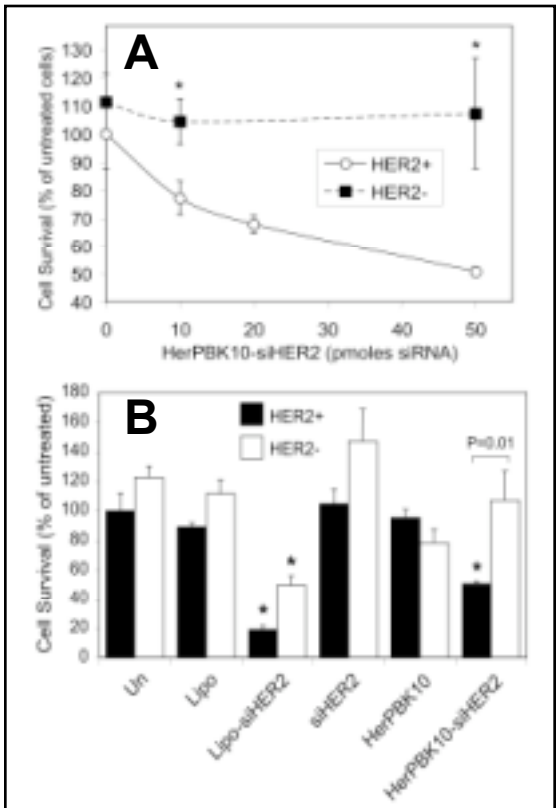

Fig. 3. Survival (assessed by crystal violet stain) of MDA-MB-435 (HER2+) and MDA-MB-231 (HER2-) cells after receiving (**A**) dosed HerPBK10-siRNA conjugates, and (**B**) 50 pmoles siHER2 (anti-HER2 siRNA) by non-specific (Lipo-mediated) or targeted (3 ug HerPBK10) complexes in 100 uL complete media/well of a 96-well plate. Complexes were prepared as described in Fig. 2. Un, untreated. In **A**, *, $P<0.02$ (HER2+ vs HER2- cells). In **B**, *, $P<0.05$ (compared to untreated). Significant differences determined by 2-tailed t tests.

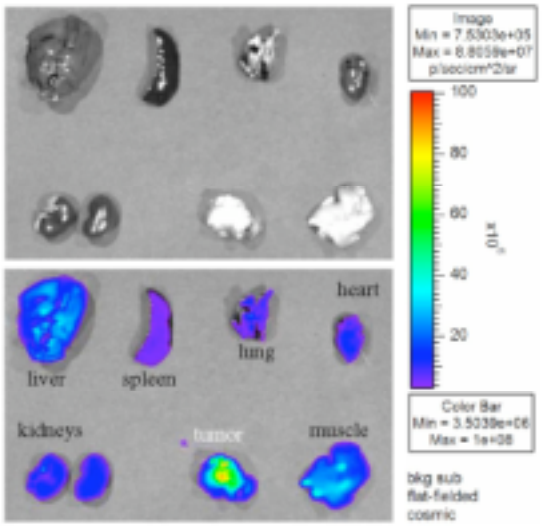

Fig 4. The 'Her' ligand preferentially targets HER2+ tumors in vivo. Tumor-bearing mice were injected with 3 nmoles of GFP-Her via the tail vein. Tissues were harvested at 3.5 h after injection and visualized using a Xenogen small-animal imager. Fluorescence signal intensity from GFP corresponds to the spectrum on the color bar (blue, low/no fluorescence; red, highest fluorescence).

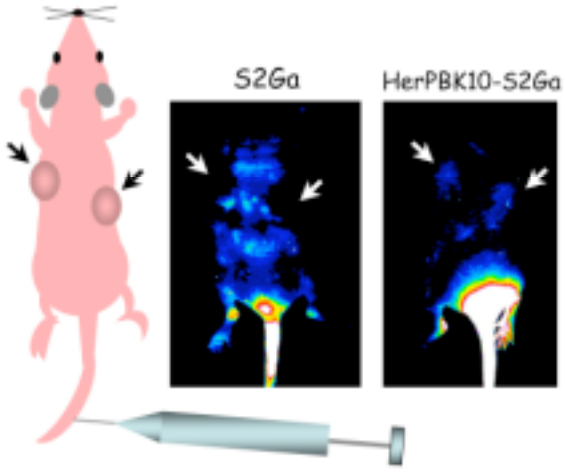

Fig. 5. In vivo tumor targeting of siRNA vehicle. Mice received i.v. injection of free fluorescent label (S2Ga) or labeled HerPBK10 (15 nmoles) and were imaged at 2.5 h post-injection using MISTI imaging system (*developed by Dr. D.L. Farkas. Nat. Biotechnol. 21:1269-71, 2003)*. Tumors are indicated by arrows. S2Ga fluorescence is indicated pseudocoloring.

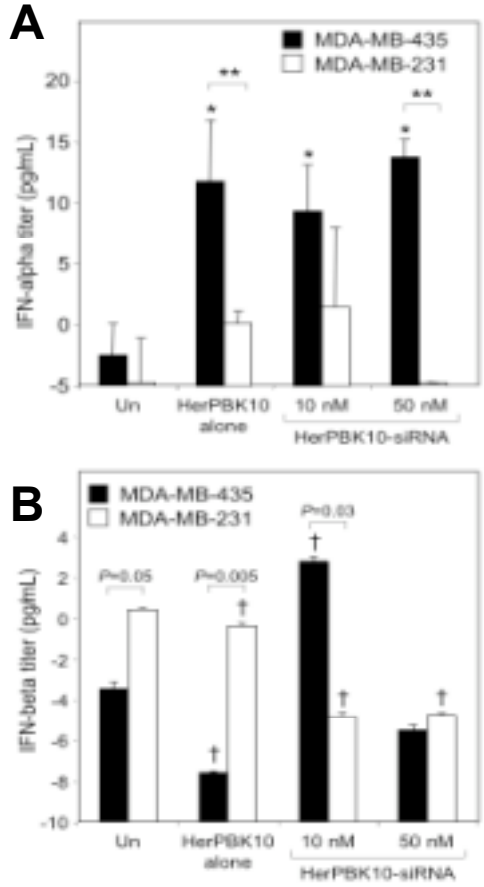

Fig. 6. Cytokine induction. Cells growing in 96-well plates ($5x10^3$ cells/well) were treated with indicated concentrations (with respect to siRNA concentration) of HerPBK10-siRNA complexes or HerPBK10 (3ug per well, at equivalent concentration to HerPBK10 in the 50nM dose of complex). At 24h after treatment, media was collected from above the cells and assayed for (A) IFN-alpha, or (B) IFN-beta titer by sandwich ELISA following manufacturer's protocol (PBL Biomedical Laboratories). *, $P<0.01$ (compared to untreated); **, $P=0.01$ (HER2+ vs HER2- cells); †, $P<0.05$ (compared to untreated). Statistical significances determined by 2-tailed t tests.